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ORIGINAL PAPER

Construction of a fosmid library of cucumber (*Cucumis sativus*) and comparative analyses of the eIF4E and eIF(iso)4E regions from cucumber and melon (*Cucumis melo*)

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Abstract A fosmid library of cucumber was synthesized as an unrestricted resource for researchers and used for comparative sequence analyses to assess synteny between the cucumber and melon genomes, both members of the genus *Cucumis* and the two most economically important plants in the family Cucurbitaceae. End sequencing of random fosmids produced over 680 kilobases of cucumber genomic sequence, of which 25% was similar to ribosomal DNAs, 25% to satellite sequences, 20% to coding regions in other plants, 4% to transposable elements, 13% to mitochondrial and chloroplast sequences, and 13% showed no hits to the databases. The relatively high frequencies of ribosomal and satellite DNAs are consistent with previous analyses of cucumber DNA. Cucumber fosmids were selected and sequenced that carried eukaryotic initiation factors (eIF) 4E and iso(4E), genes associated with recessively inherited resistances to potyviruses in a number of plants. Indels near eIF4E and eIF(iso)4E mapped independently of the *zym*, a recessive locus conditioning resistance to *Zucchini yellow mosaic virus*, establishing that these candidate genes are not *zym*. Cucumber sequences were compared with melon BACs carrying eIF4E and eIF(iso)4E and revealed extensive sequence conservation and synteny between cucumber and melon across these two independent genomic regions. This high degree of microsynteny will aid in the cloning of orthologous genes from both species, as well as allow for genomic resources developed for one *Cucumis* species to be used for analyses in other species.

Keywords *Cucumis sativus* · *Cucumis melo* · Potyvirus resistance · Eukaryotic initiation factors · Synteny

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Names are necessary to report factually on available data; however, the US Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

Comparative-genomic analyses have revealed synteny among closely related species for positions and functions of orthologous genes (Keller and Feuillet 2000; Paterson et al. 2000). High levels of synteny allow genetic information from well-characterized genomes to be used for gene tagging and isolation in less studied species. Cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.) are the most economically important plants in the genus *Cucumis*. Both cucumber and melon are diploids with relatively small nuclear genomes at 0.38 and 0.47 pg per 1C, respectively (Bennett and Leitch 2004); however, they belong to distinct subgenera [*Cucumis* and *Melo*, respectively (Kirkbride 1993)] and possess different chromosome numbers [$2n = 2 \times = 14$ for cucumber and $2n = 2 \times = 24$ for melon (Robinson and Decker-Walters 1997)]. Although there has

been no extensive comparative-genomic analysis of cucumber and melon, there is evidence for sequence conservation (Neuhausen 1992; Katzir et al. 1996; Danin-Poleg et al. 2000) and synteny near the *zym* locus (Park et al. 2004).

Potyviruses are important viral pathogens that are especially destructive in the Cucurbitaceae (Provvidenti et al. 1984; Luis et al. 1998; Yuki et al. 2000). Sources of potyvirus resistances have been identified in the cucurbits and often are recessively inherited, such as the *prsv-1* (Wang et al. 1984) and *zym* (Provvidenti 1987; Kabelka et al. 1997) loci of cucumber conditioning resistance to the watermelon strain of *Papaya ringspot virus* (PRSV-W) and *Zucchini yellow mosaic virus* (ZYMV), respectively. The cucumber population 'TMG1' possesses allelic or tightly linked recessive resistances to *Moroccan watermelon mosaic virus*, *Watermelon mosaic virus*, *Zucchini yellow fleck virus*, and ZYMV (Wai et al. 1997, Grumet et al. 2000, Park et al. 2000). Recessively inherited resistances increase the time and effort to breed resistant cultivars because a selfing generation is required during backcrossing to identify plants carrying the recessive allele. The recessive potyvirus resistances are occasionally conditioned by mutations in eukaryotic translation initiation factors (eIF)4E and eIF(iso)4E (Ruffel et al. 2002, 2005, 2006; Nicaise et al. 2003; Kang et al. 2005; Kanyuka et al. 2005; Sato et al. 2005; Stein et al. 2005). These plant proteins are intimately associated with recognition and translation of the potyviral RNA (Díaz-Pendón et al. 2004; Robaglia and Caranta 2006; Maule et al. 2007). If potyvirus resistances were syntenic among the cucurbits, molecular markers flanking a resistance locus in one species could be used in marker-assisted breeding for other cucurbits. The goals of this research were to develop a publicly available genomic library of cucumber, assess the level of microsynteny across orthologous regions of the cucumber and melon genomes carrying eIF4E and eIF(iso)4E, and to evaluate these genes as candidates for the *zym* locus of cucumber.

Materials and methods

Construction and characterization of a cucumber fosmid library

Lyophilized tissue of cucumber cultivar 'Straight 8' (ST8) was provided to Warwick Plant Genomic Libraries (Warwick UK). Randomly sheared DNA from ST8 nuclei was purified after size fractionation by gel electrophoresis, end-polished, and cloned into the *Eco*72I blunt-end site of the pCC2FOS vector as described by the manufacturer (Epicentre Technologies, Madison, WI USA). The fosmids were packaged using the MaxPlax Packaging Extract (Epicentre) and shipped absorbed to competent bacteria (TransforMax EPI300 from Epicentre). Aliquots were plated on LB plates with chloramphenicol (12.5 μg/ml) and used to calculate titers and assess library quality. The remaining bacteria with absorbed fosmids were shipped to Clemson University Genomics Institute (CUGI) for plating, random picking, and arraying into high-density plates. Double-spotted, high-density filters were produced using standardized techniques at CUGI.

Five hundred 76 colonies were randomly picked and bacteria were grown overnight in 5 ml LB with 50 μg/ml chloramphenicol. Fosmid copy control was released by incubation at 37°C for 5 h with shaking at 300 rpm in horizontal 2 ml tubes containing 1.2 ml of LB with 50 μg/ml chloramphenicol, 300 μl of the overnight culture, and 1.5 μl of Copy Control Induction Solution (Epicentre). The tubes were then centrifuged for 1 min and the supernatant discarded. Fosmids were isolated using a modified protocol of the QIAgen (Valencia, CA, USA) plasmid minipreparation. The bacteria were re-suspended in 250 μl of buffer P1 and vortexed. Lysis buffer P2 (250 μl) was added and the tubes were inverted six times. Neutralization buffer N3 (350 μl) was added and the tubes were inverted six times. The tubes were placed on ice for 20 min. and then centrifuged at 13,000 × *g* for 10 min at 4°C. The liquid was transferred to a new tube and centrifuged for 10 min. The DNA was precipitated after addition of 600 μl of isopropanol, incubation on ice for 10 min, and centrifugation at maximum speed in a microfuge for 20 min at 4°C. The pellets were washed once with 200 μl of PE buffer. DNAs were allowed to dry for 20 min and resuspended in 30 μl of elution buffer (EB), and incubated at 37°C for 1 h. Fosmids were end-sequenced from both directions using primers to the vector (5′-GTACAACGACACCTAGAC and 5′- CAGGAAACAG CCTAGGAA). Sequence reactions had 3 μl of the fosmid DNA, 2 μl Big Dye (ABI, Foster City, CA, USA), 3 μl Big Dye Buffer, and 20 pmol of primer in a final volume of 20 μl. Cycling conditions one cycle at 95°C for 2.5 min, followed by 99 cycles at 96°C for 25 s, 55°C for 20 s and 62°C for 5 min. Reactions were cleaned with Clean Seq beads according to manufacturer's (Agencourt, Beverly, MA, USA) directions. Vector and trash sequences were removed using Sequencer (Genecodes, Ann Arbor MI, USA). Nucleotide searches were performed using blastn against NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST) and translated searches were performed using BLASTX (Altschul et al. 1997) against the UniProt (Apweiler et al. 2004) uniref100 database (http://www.pir.uniprot.org/) to classify sequences as ribosomal, satellite, mitochondrial, chloroplast, putatively coding, or unique at <10e-4.

Isolation of cucumber fosmids carrying 4E and (iso)4E

Genomic amplicons of eIF4E and eIF(iso)4E from melon were produced using degenerate primers (5′-TGGACITT

YTGGTTYGAYAA and 5′-GRRTCYTCCCAYTTIGGY TC) (Nieto et al. 2006). PCR was performed with an annealing temp of 55°C for 20 s and an extension time of 2 min. Two amplicons were produced, 1.9 kb for eIF4E and 500 bp for eIF(iso)4E, and were purified, cloned after TA tailing, and sequenced to confirm identities. Cloned amplicons were hybridized to high-density filters carrying cucumber fosmids as described by CUGI (http://www.genome.clemson.edu/protocols/hyb_filter.shtml). Fosmids showing strong signals were selected, grown, and minipreped and production of target amplicons confirmed by PCR.

The fosmids carrying eIF4E (175K21) and eIF(iso)4E (147D19) were sequenced after transposon tagging using the EZ-Tn5™ <*ori*V/KAN-2> Insertion Kit according to the manufacturer's (Epicentre) recommendations. Sequences were trimmed and assembled using Sequencher. Primers were designed based on sequences at the ends of large contigs and were tested for amplification from the fosmid clones. Amplicons were cloned into the pGEM T-easy vector and transformed into bacteria according to the manufacturer's (Promega) recommendations. White colonies were selected and grown in LB with ampicillin overnight at 37°C. Plasmids were isolated using the QIAprep Spin Miniprep Kit according to manufacturer's directions and inserts sequenced using primers to the vector.

Isolation and sequencing of a melon BAC carrying eIF(iso)4E

A cDNA of eIF(iso)4E from *Arabidopsis thaliana* (Duprat et al. 2002) and was hybridized to filters of the melon BAC library (Luo et al. 2001). Three BACs with strong signals were selected, fingerprinted using restriction enzymes, and aligned. One BAC (107H02) was sequenced. BAC cultures were purified using a modified protocol of the QIAgen Spin Prep Kit described for the fosmid miniprep except that 4 ml of culture were harvested. The isolated BAC was sheared and fragments subcloned using the TOPO Shotgun Sub-Cloning Kit according to the manufacturer's (Invitrogen) protocol. Sublcones were sequenced using primers to the vector as described previously.

Sequence analyses and annotation

BAC and fosmid sequences were assembled using Sequencher. Gaps in contigs were closed by designing primers from the ends and production of amplicons across the gaps. Amplicons were sequenced and added to the contigs. The contig sequences were initially analyzed by BLASTX (Altschul et al. 1997; http://www.ncbi.nlm.nih.gov/BLAST/), GENESCAN (Burge and Karlin 1997; http://www.genes.mit.edu/GENSCAN.html), and FGENESH (http://www.softberry.com). Putative proteins and similarities were identified using TBLASN and BLASTP (Altschul et al. 1997) and Blast2Sequences (Tatusova et al. 1999; http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Genomescan (Yeh et al. 2001) was used to refine gene predictions based on previously annotated structures. Reports on annotated genes were made by recording the protein size, position on the contig, number of hits using TBLASTN at NCBI at <10e-4, and the accession number, organism, and evalue of the best hit to the protein sequence. Similarities between cucumber and melon genomic sequences were compared using 100 bp windows with the LAGAN (Brudno et al. 2003) alignment program in mVista (http://www.genome.lbl.gov/vista/index.shtml).

Mapping of eIF4E and eIF(sio)4E in cucumber

Copy number of eIF4E and eIF(iso)4E in the cucumber and melon genomes were estimated by Southern hybridizations (Kennard et al. 1994) using DNAs from TMG1 and ST8, parents of a cucumber mapping family (Park et al. 2000), and 'T111' Piel de Sapo and PI 161375, parents of a melon mapping family (Gonzalo et al. 2005). Polymorphisms were identified in genomic regions flanking eIF4E and eIF(iso)4E utilizing primers designed from contigs. Amplicons from TMG1 and ST8 were gel extracted, sequenced, and aligned with the fosmid sequence. When a polymorphism was detected, new primers were designed surrounding the polymorphism to produce amplicons of 500 bp or less. Primers LP-CTAAGCGGGATGGAATCAAG and RP-TCAACCACATGGAA CCACAT amplified polymorphisms for mapping of eIF4E and primers LP-CCAAGC ACTTGGATTTAGTCG and RP-TGAAATGGTTTG CCCAACTT for eIF(iso)4E in cucumber. PCR was performed with 35 cycles of an annealing temp of 55°C for 20 s and an extension time of 1 min 10 s. Amplicons were resolved on 3–4% (w/v) Super Fine Resolution agarose (Amresco, Solon, OH, USA). Amplicons from each RIL were gel extracted, cloned, and sequenced to confirm genotypes. Markers near eIF4E and eIF(iso)4E were added to the previously described linkage map for the TMG1 and ST8 (Park et al. 2000) using MapManager (Manley et al. 2001) at $P < 0.0001$ and the Kosambi mapping function.

Results

Construction and characterization of a cucumber fosmid library

We synthesized a fosmid library of cucumber as an unrestricted resource for researchers. Initially 99,840 random fosmids were robotically picked and arrayed into 260 384-well plates. The average insert was 38 kb, corresponding to

4.3× coverage of the cucumber genome. Although this number of fosmids represents relatively low genome coverage, we used this initial library to assess the usefulness of fosmid clones for genomic cloning and sequencing and it is our intention to double the number of fosmids for more complete genome coverage.

We produced 1,046 random end sequences (Genbank accessions ET024028 to ET025073) to yield 680,248 bp of random genomic sequence from cucumber. Translated and nucleotide searches revealed the best hit for one end per fosmid (576 ends total), of which 24.6% showed significant similarities to ribosomal DNAs, 23.3% to the predominant satellite sequences, 22.1% to coding regions in other plants, 4.2% to transposable elements, and 7.0 and 5.4% to mitochondrial and chloroplast sequences, respectively (Fig. 1). A total of 13.4% of the fosmid ends showed no significant (<10e-4) hits to the databases.

Comparative analyses of the eIF4E and eIF(sio)4E genomic regions in cucumber and melon

The eIF4E and eIF(iso)4E genes in cucumber and melon are low copy (Fig. 2). We isolated and sequenced cucumber fosmids carrying eIF4E (175K21) and eIF(iso)4E (147D19), as well as a melon BAC (107H02) carrying eIF(iso)4E. A 92-kb melon BAC (1-21-10) carrying eIF4E was previously sequenced and annotated (Genbank accession EF188258) (Nieto et al. 2006). Sequences of the cucumber fosmids carrying eIF4E and eIF(iso)4E are available as Genbank accessions EU196160 and EU232173, respectively; the melon BAC region carrying eIF(iso)4E is accession EU232172. Amino-acid sequences of the eIF4E and eIF(iso)4E genes were 46% similar in cucumber and 49% similar in melon, in agreement with previous estimates of sequence similarity between these two genes

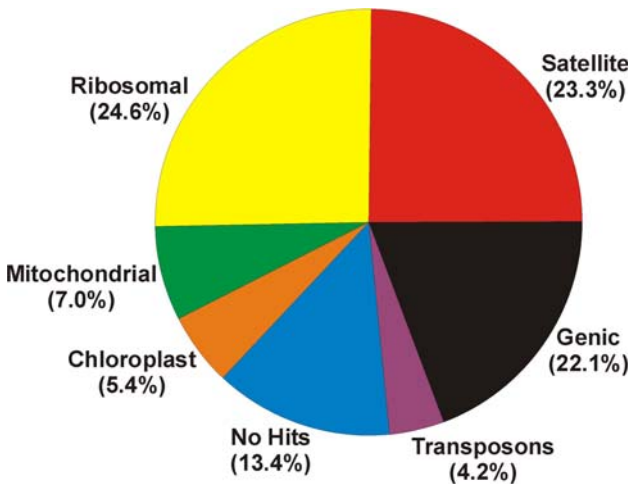

Fig 1 Distribution of random single-end sequences from the fosmid library of cucumber

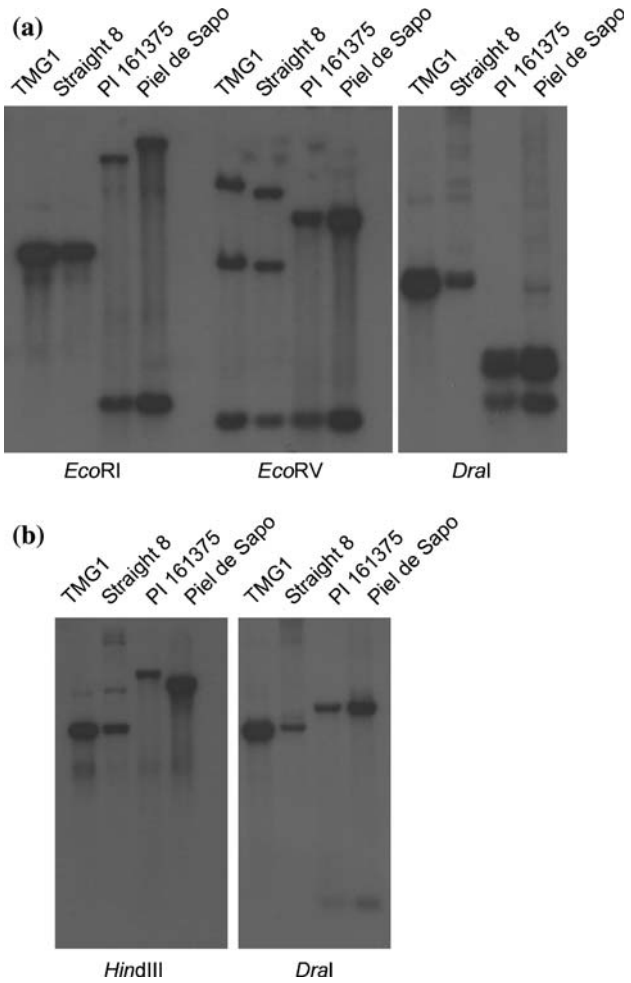

Fig. 2 Autoradiograms from hybridizations of eIF4E (**a**) and eIF(iso)4E (**b**) to DNA-gel blots of cucumber (TMG1 and Straight 8) and melon (Piel de Sapo 'T111' and PI 161375)

(Ruffel et al. 2004). The genomic regions flanking these genes in the same species showed essentially no similarities, as expected because the eIF4E and eIF(iso)4E genes arose from a relatively ancient duplication (Duprat et al. 2002).

Significant sequence similarities and microsynteny were revealed between the melon BAC and cucumber fosmid carrying eIF4E, as well as those carrying eIF(iso)4E (Fig. 3; Table 1). For eIF4E genomic regions in cucumber and melon, Genescan, Genomescan, and BLAST searches revealed three conserved putative coding regions (a hypothetical protein, exonuclease, and eIF4E) in the same orientations, sizes, and number of exons (Fig. 3). The relative positions of these orthologous genes were similar except for the presence of a gypsy-like retrotransposon and an adjacent hypothetical protein in cucumber (Table 1; Fig. 3). Putative exon sequences from the cucumber eIF4E fosmid (excluding retrotransposon and adjacent hypothetical protein) were 95% similar to the

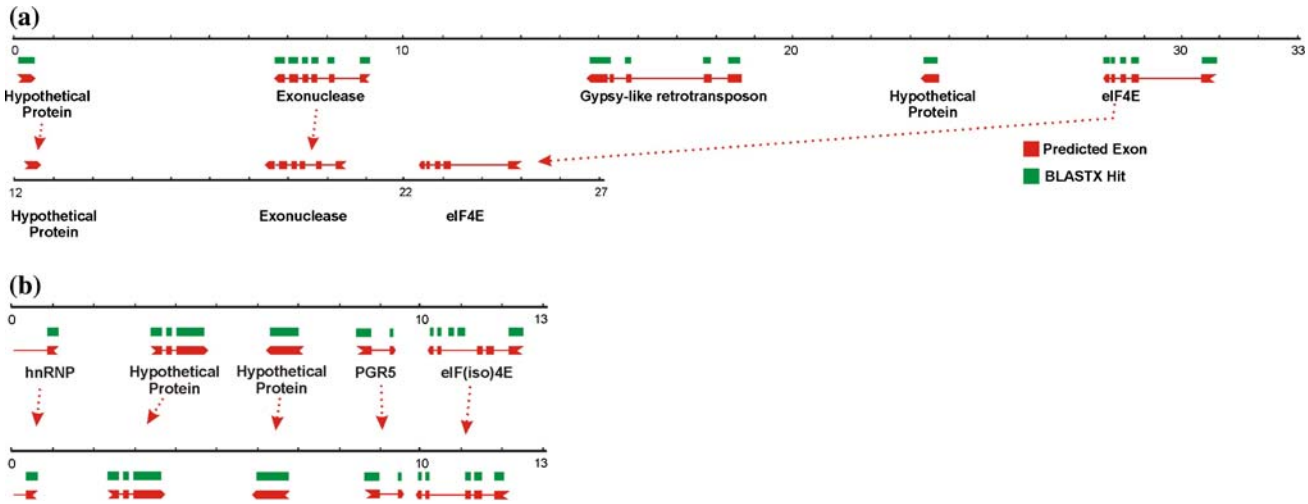

Fig. 3 Annotation and similarities among genomic regions of cucumber (*above*) and melon (*below*) carrying eIF4E (**a**) and eIF(iso)4E (**b**). Only part of the melon BAC sequences are shown

Table 1 Locations and similarities of predicted genes from cucumber fosmids 175K21 and 147D19 and melon BAC 107H02

Gene	Number of AA[a]	Position in bp[b]	Number of hits[c]	Genbank accession	Best hit organism	*E* value
Cucumber fosmid 175K21						
Hypothetical protein	145	93–530	116	EF188258	*C. melo*	3e-45
Exonuclease	336	9,016–6,641	109	NM_00184155	*Arabidopsis thaliana*	2e-141
Gypsy-like retrotransposon	377	18,559–14,757	2,731	DQ023670	*S. latifolia*	2e-54
Hypothetical protein	103	23,664–23,353	108	AM440031	*V. vinifera*	2e-6
eIF4E	235	28,037–30,843	117	DQ393832	*C. melo*	1e-118
Cucumber fosmid 147D19						
Putative HnRNP[d]	95	1,046–15	211	AK226323	*A. thaliana*	4e-28
Hypothetical protein	277	3,385–4,430	32	NM_128900	*A. thaliana*	1e-16
Hypothetical protein	288	7,418–6,552	105	AM476678	*V. vinifera*	4e-90
PGR5	127	8,841–9,743	44	NM_126585	*A. thaliana*	2e-37
eIF(iso)4E	203	12,170–10,195	103	DQ022082	*C. annuum*	2e-86
Melon BAC 107H02						
Putative HnRNP[d]	76	556–326	210	AK226323	*A. thaliana*	4e-28
Hypothetical protein	277	2,548–3,643	47	NM_128900	*A. thaliana*	6e-15
Hypothetical protein	288	6,697–5,831	101	AM476678	*V. vinifera*	4e-90
PGR5	127	8,674–9,498	44	NM_126585	*A. thaliana*	2e-37
eIF(iso)4E	203	11,914–9,935	103	AY699609	*N. tabacum*	1e-85

[a] *AA* amino acids

[b] *bp* basepairs

[c] *E* value <1e–10 for searches to NCBI databases restricted to plant species

[d] Partial sequence from end of contig

predicted coding sequences on the eIF4E melon BAC; 70% of putative noncoding regions (excluding the retroviral insertion) near eIF4E on the cucumber fosmid and melon BAC showed >70% sequence similarity (Fig. 4). For the eIF(iso)4E regions in cucumber and melon, five putative coding regions included a putative HnRNP (heterogeneous nuclear ribonucleoprotein), two hypothetical proteins, PGR5 (proton gradient regulation), and eIF(iso)4E, showed the same orientations, number of exons, and relative sizes (Table 1; Fig. 3). Putative exon sequences on the eIF(iso)4E cucumber fosmid were 97% similar to exons on the eIF(iso)4E BAC from melon and 80% of the noncoding sequences surrounding eIF(iso)4E showed >70% sequence similarity (Fig. 4).

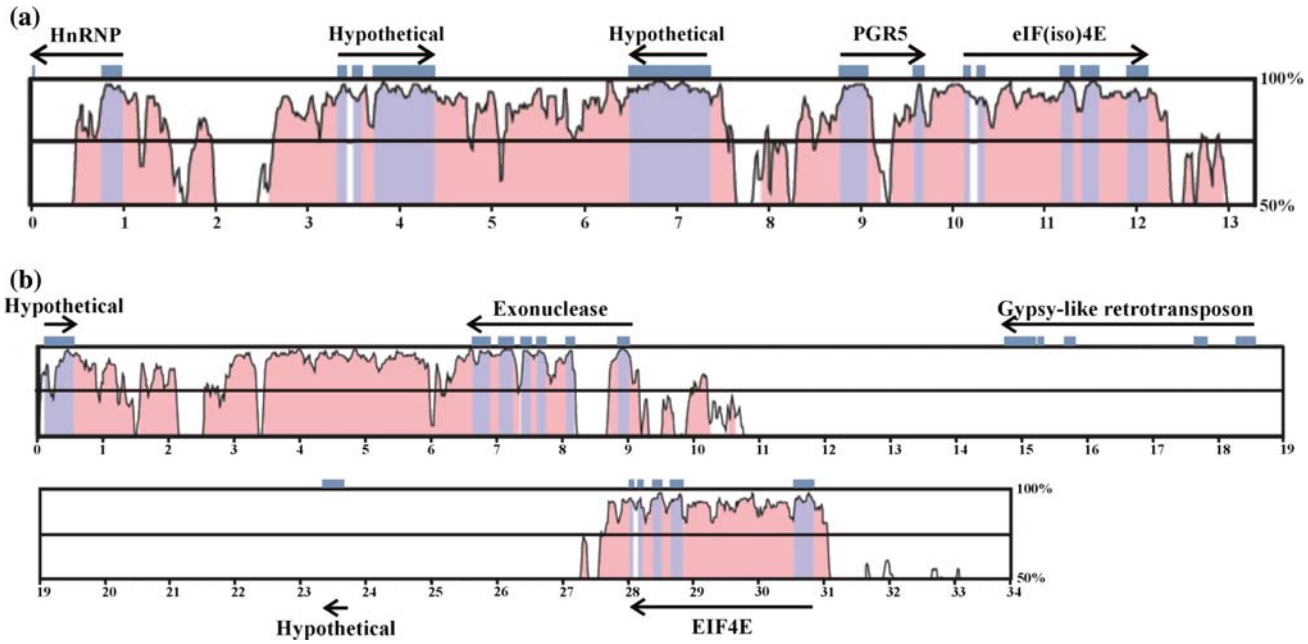

Fig. 4 Vista similarity plot for the eIF(iso)4E (**a**) and eIF4E (**b**) genomic regions from cucumber and melon. Sizes in kilobases are shown at *bottom*. Percent similarities are shown on the *right border*. Introns and intergenic regions are shown in *pink*, exons in *blue*. Gene names and orientations are designated with *arrows*

Mapping of eIF4E and eIF(iso)4E in cucumber

EIF4E and eIF(iso)4E are associated with recessively inherited virus resistances in numerous plants (Robaglia and Caranta 2006). We previously generated F_6 recombinant inbred lines (RILs) from a cross between ST8 and TMG1 and scored the RILs for reactions to ZYMV (Park et al. 2000). We evaluated eIF4E and eIF(iso)4E in cucumber as candidates for the cluster of recessive potyvirus resistances in TMG1. We were not able to identify any polymorphisms in the coding regions or introns of the eIF4E and eIF(iso)4E genes in cucumber, likely due to the extremely low level of DNA polymorphism in cucumber (Kennard et al. 1994; Dijkhuizen et al. 1996). We therefore exploited the cucumber fosmid library in order to identify DNA polymorphisms in genomic regions in the adjacent eIF4E and eIF(iso)4E coding regions. For both genes, we designed primers in genomic regions flanking eIF4E and eIF(iso)4E. For eIF4E, we identified a 20-bp indel upstream from this gene that was polymorphic between ST8 and TMG1 (accession numbers BV725490 and BV725489, respectively). This indel cosegregated with AFLPs E25/M47-F-206 and E16/M49-F-138 and mapped to linkage group DE (Fig. 5). We identified a 12-bp indel near eIF(iso)4E (accession numbers BV725488 and BV725487 for ST8 and TMG1, respectively) and this polymorphism mapped to linkage group P (Fig. 5). Both markers segregated independently of the *zym* locus on linkage group Q (Park et al. 2000).

Discusssion

We synthesized and characterized the first fosmid library of a cucurbit. Although BAC libraries of cucumber have been constructed (Nam et al. 2005), the developers were unwilling to make this resource available to other researchers. Advantages of fosmids over BACs include high cloning efficiencies with no restriction-enzyme bias, copy control that maintains single-copy clones in bacteria to ensure insert stability with no selection against toxic gene products, induction to high-copy numbers, and relatively easy minipreparations and sequencing. The library, individual fosmids, and double-spotted high-density filters are available without restriction on a cost-recovery basis from the Clemson University Genomics Institute (http://www.cugi.org). Approximately 50% of fosmid clones carried rDNA or satellite sequences (Fig. 1). The relatively high frequencies of ribosomal and satellite DNAs are consistent with previous analyses of cucumber DNA. Heterochromatic regions comprise approximately 50% of the cucumber nuclear genome (Ramachandran et al. 1985). The ribosomal coding regions cover about 10% of the nuclear DNA (Hemleben et al. 1982) with 45s components concentrated on chromosomes 1, 2, and 5 (Hoshi et al. 1999). The satellite DNAs are divided into three main fractions (I/II, III, and IV) and comprise approximately 30% of the nuclear DNA (Ganal et al. 1986; Ganal and Hemleben 1988). The relatively low frequencies of mitochondrial and chloroplast DNAs were likely due to successful isolation of nuclei prior to shearing and cloning of genomic fragments into the

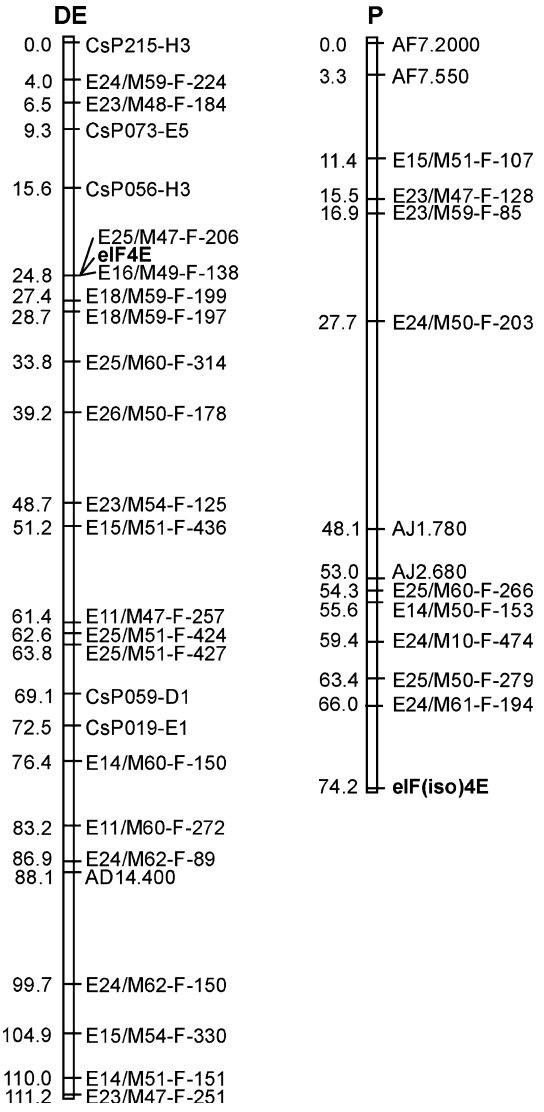

Fig. 5 Cucumber linkage groups DE and P carrying eukaryotic initiation factors eIF4E and eIF(iso)4E, respectively

fosmids. Although the cucumber genome is approximately twice the size of *A. thaliana* (Bennett and Leitch 2004), the relatively high proportion (~50%) of ribosomal and satellite DNAs indicates that the cucumber genome should possess regions of relatively high gene densities, facilitating map-based cloning of target genes.

Although eIF4E and eIF(iso)4E were not linked to the cluster of recessive potyvirus resistance loci in TMG1, their placement on the cucumber map (Fig. 5) will be useful for assessing their relationships with other virus resistances. This study validates the approach to exploit the fosmid library to identify polymorphisms in genomic regions adjacent to candidate genes for mapping within the narrow genetic background of cucumber. Our results revealed extensive sequence conservation and synteny between cucumber and melon across two independent genomic regions; both species possessed the same genes in the same orientation, except for the retroviral insertion near the eIF4E gene in cucumber (Table 1; Fig. 3). Noncoding regions were also highly similar between cucumber and melon (Fig. 4). Although these two *Cucumis* species are distantly related and possess different chromosome numbers (Kirkbride 1993), strong microsynteny and sequence similarities will aid in the cloning of orthologous genes from both species, as well as allow for genomic resources developed for one *Cucumis* species to be used for analyses in other species.

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